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Evolution of Developmental Control Mechanisms

The function of Notch signalling in segment formation in the crustacean *Daphnia magna* (Branchiopoda)

Bo Joakim Eriksson [a,b], Petra Ungerer [a], Angelika Stollewerk [a,*]

[a] School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK
[b] Department of Neurobiology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

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ABSTRACT

Ten years ago we showed for the first time that Notch signalling is required in segmentation in spiders, indicating the existence of similar mechanisms in arthropod and vertebrate segmentation. However, conflicting results in various arthropod groups hampered our understanding of the ancestral function of Notch in arthropod segmentation. Here we fill a crucial data gap in arthropods and analyse segmentation in a crustacean embryo. We analyse the expression of homologues of the *Drosophila* and vertebrate segmentation genes and show that members of the Notch signalling pathway are expressed at the same time as the pair-rule genes. Furthermore, inactivation of Notch signalling results in irregular boundaries of the *odd-skipped-like* expression domains and affects the formation of segments. In severe cases embryos appear unsegmented. We suggest two scenarios for the function of Notch signalling in segmentation. The first scenario agrees with a segmentation clock involving Notch signalling, while the second scenario discusses an alternative mechanism of Notch function which is integrated into a hierarchical segmentation cascade.

Introduction

In 2003 we could show for the first time that Notch signalling, which is required for mesoderm segmentation in vertebrates (Morales et al., 2002), is also involved in segment formation in spiders (Stollewerk et al., 2003). This was the first evidence that similar mechanisms of segment formation operate in long-diverged phyla such as arthropods and vertebrates. Follow-up papers showed that members of the Notch signalling pathway are expressed during embryonic segmentation in two additional arthropod groups, insects and myriapods (Chipman and Akam, 2008; Kainz et al., 2011; Mito et al., 2011; Pueyo et al., 2008). However, there is disagreement on the function of Notch signalling in arthropod segmentation. Some publications show that segments are missing in Notch loss-of-function experiments (Mito et al., 2011; Schoppmeier and Damen, 2005) but it was argued that this phenotype might be due to early functions of Notch, for example in caudal lobe formation, or pleiotropic functions that culminate in the loss of segments (Kainz et al., 2011; Oda et al., 2007). Furthermore, some insects do not require Notch signalling in segmentation and functional studies are missing in myriapods.

This raises the question of whether Notch signalling is involved in segmentation in all arthropods and if so, whether it fulfils similar functions. Here we address this question, by filling a critical data gap in arthropods and analysing the molecular processes of segmentation in a crustacean embryo, the water flea *Daphnia magna*.

Arthropods, including crustaceans, show a range of short to long germ development. In long germ development, the cell material for the formation of embryonic segments is present at the start of germ band formation and segments are formed simultaneously (e.g. *Drosophila*), while in short and intermediate germ development segments are formed partially sequentially from cell material derived from a posterior growth zone (e.g. *Daphnia*). In crustaceans, the cellular processes of segmentation have been analysed in great detail in malacostracans, which form segments by asymmetric divisions of teloblasts, except for amphipods (Scholtz and Wolff, 2013). Each synchronous division of the posterior ectoteloblasts generates a row of cells which divide further to generate the four rows constituting a parasegment (Martinez-Arias & Lawrence 1985; Scholtz & Dohle 1988 in crayfish, grasshopper Patel & Goodman). However, teloblasts seem to represent an apomorphic character of malacostracans (Fischer et al., 2010; Scholtz and Wolff, 2013). In branchiopods, mitotic divisions are scattered over the whole area of the posterior growth zone and elongation of the germ band occurs mainly by intercalation (Manzanares et al., 1993). In representatives of all major crustacean groups (e.g. branchiopods, malacostracans, and cephalocarids), the anterior-most head segments show a precocious development and so-called nauplius larvae hatch from the eggshells, which typically only consist of three head segments

* Corresponding author. Fax: +44 20 7882 7732.
E-mail address: a.stollewerk@qmul.ac.uk (A. Stollewerk).

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and a posterior growth zone (e.g. the branchiopod *Artemia*) (Scholtz and Wolff, 2013). Thus, posterior growth and segmentation mainly occur in larval stages. In crustacean embryos that show direct development, such as the malacostracan *Orchestia cavimana* (Scholtz and Wolff, 2013) or the branchiopod *Daphnia* (as shown in this study), a nauplius-like stage can be observed in the egg ('egg nauplius'), i.e. the anterior head segments differentiate precociously and develop appendages before the postnaupliar segments are formed (Scholtz, 2000).

In *Drosophila* three classes of genes regulate segmentation in a hierarchical, temporal order—the gap genes, the pair-rule genes and the segment polarity genes (Schroeder et al., 2011). While the expression domains of the gap genes cover large areas that develop into several segments, the pair-rule gene expression in alternating transverse stripes represents the first sign of segmental subdivision of the *Drosophila* embryo. The segment polarity genes confer anterior–posterior identity within a segment. The segmentation genes have been named after their mutant phenotype in *Drosophila*. However, their functions and interactions have considerably diverged in arthropods, in particular regarding the pair-rule genes (Choe and Brown, 2007; Peel et al., 2005). Henceforth, we therefore refer to homologies in terms of sequence rather than function/expression, when using the terms 'pair-rule' or 'gap genes'.

Information on the molecular processes of segmentation in crustaceans is fragmentary. The expression patterns of a few homologues of *Drosophila* segmentation genes have been published in representatives of malacostracans and branchiopods (e.g. the gap gene *hunchback*, the pair-rule gene *Pax 3/7*, and the segment polarity gene *engrailed*); however, their roles in segmentation remain unclear with the possible exception of the segment polarity gene *engrailed* (*en*) (Davies et al., 2005; Kontarakis et al., 2006; Manzanares et al., 1993; Patel et al., 1989).

Here we present the expression patterns of homologues of the *Drosophila* segmentation genes in relation to segment formation and show that members of the Notch signalling pathway are expressed at the same time as pair-rule genes. Furthermore, we demonstrate that Notch signalling is involved in the formation and patterning of segments.

Materials and methods

DAPT treatment

D. magna embryos were collected at 0 to 8 h of development (stages 0–5), before the appearance of antennal segment 2 (stage 6.1; see Fig. 1C), which is the first morphological landmark in living, unstained embryos. At stage 6.1, *D. magna* embryos develop a vitelline membrane, which prevents the penetration of DAPT (2,5-bis[4-dimethylaminophenyl]-1,3,4- thiadiazole, Sigma). We incubated the embryos in 5-well plates in 0.75 mM DAPT (diluted from a 10 mM stock solution in DMSO) in mineral water for 4 h at 25 °C. DAPT concentrations below 0.75 mM did not result in statistically relevant numbers of embryos showing a phenotype. Control embryos were incubated in an equal volume of DMSO in mineral water for the same time. Subsequently embryos were transferred to a 5-well plate containing *Daphnia* medium, and left to further develop overnight. All embryos within a well were fixed at the same time (up to 100 embryos per well), so that we obtained a mixture of various stages. Embryos, which were older

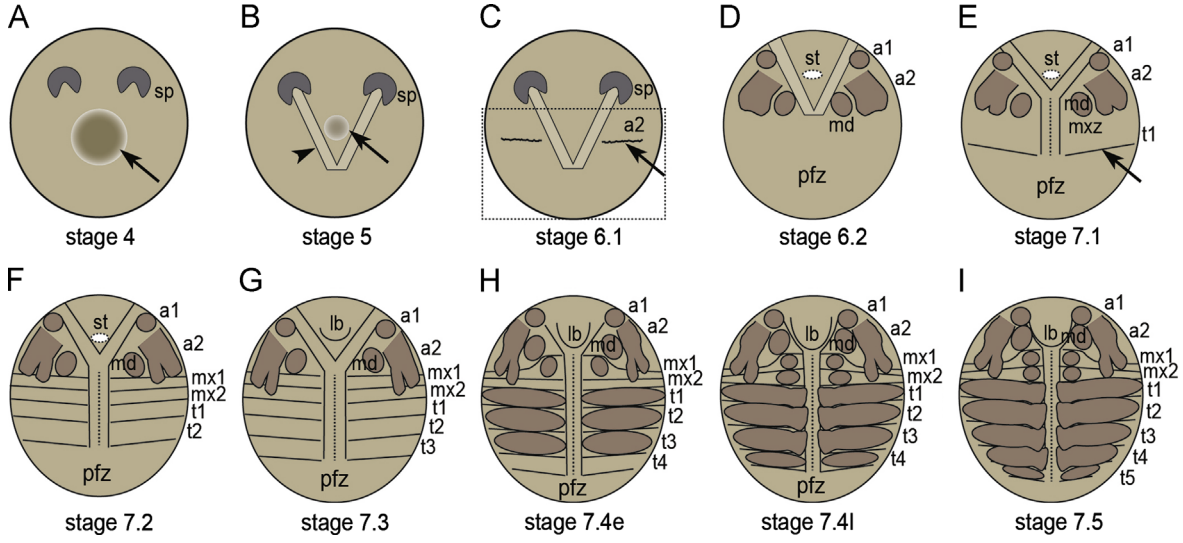

Fig. 1. (A–H) Formation of the embryonic segments in *Daphnia magna*. The schematic drawings are based on a staging system that relies on morphological landmarks (unpublished results). Each stage shown takes about 2 2.5 h, except for stage 7.4. Light brown V-shaped area: developing neuroectoderm corresponding to the naupliar segments; middle brown: background colour indicating the shape of the *Daphnia* embryo; dark brown: limb anlagen; grey half-moons: Scheitelplatten; transverse lines: morphologically visible segment borders; Y-shaped area: developing central nervous system; dashed vertical line: ventral midline; white oval: stomodeal invagination; half-circle: labrum. (A) At stage 4, bilateral 'Scheitelplatten' (sp) are visible anterior to the gastrulation zone (arrow). (B) At stage 5, the V-shaped naupliar neuroectoderm forms (arrowhead). (C) Paired diagonal furrows corresponding to the border of the limb buds of the second antennal segment are visible at stage 6.1 (arrow). The rectangle indicates the area, which is located in the anterior-most position in the scheme shown in D. (D) At stage 6.2, the limb buds of a1 and md are formed and the a2 appendages are clearly visible; pfz: postnaupliar formation zone. (E) At stage 7.1, an intersegmental furrow demarcates the posterior border of t1. The anterior border is not visible yet. The intersegmental furrow does not cross the medial neuroectodermal area. The maxillary zone (mxz) is located posterior to the mandibular segment; however, the region is not separated into mx1 and mx2 yet. (F) At stage 7.2, t2 becomes morphologically visible and intersegmental furrows separate the maxillary segments and the first thoracic segment. (G) At stage 7.3, t3 has formed. (H) Stage 7.4 takes about twice as long as the remaining stages shown here. We therefore subdivided this stage into stage 7.4 early (stage 7.4e) and stage 7.4 late (stage 7.4l). At stage 7.4e, t4 is visible and the elevated limb anlagen can be distinguished in the lateral ectoderm of mx1 and t1 to t3. At stage 7.4l, the limb buds of mx2 and t4 appear. The limb anlagen gradually separates from the lateral ectoderm during the second half of embryogenesis. (I) At stage 7.5, t5 is present and its limb anlagen can already be distinguished. This indicates that the last thoracic segment that is formed during embryogenesis shows an accelerated development compared to the remaining embryonic segments. During late embryogenesis the abdominal anlage forms which gives rise to an undetermined number of segments (data not show).

(stages 7.4–7.5) at the time of fixation, showed milder phenotypes. This correlates with the formation of the vitelline membrane during DAPT treatment, which prevents penetration of DAPT and thus correlates with a shorter exposure.

Staining and sequences

Nuclei staining and in situ hybridisation were performed as described before (Ungerer et al., 2011). Sequence data have been deposited in the European Nucleotide Archive (accession numbers: HF913441 (*Dam hunchback*); HF913442 (*Dam paired IIIA*); HF913443 (*Dam paired IIIB*)) and GenBank (accession number: HQ398105.1 (*Dam odd-skipped-like*)).

Results

Order of expression of homologues of the Drosophila segmentation cascade

In order to establish if and at which stage Notch signalling might be involved in segmentation in *D. magna*, we first established the sequence of formation of morphologically visible segments and analysed the expression patterns of homologues of the *Drosophila* segmentation cascade. In *D. magna* embryos, the five anterior segments and the protocerebral anlage develop into the head. In addition, five leg-bearing thoracic segments are generated during embryogenesis. Two staging systems have been published for three different *Daphnia* species (*Daphnia galatea*, *Daphnia hyalina*, and *Daphnia pulex*) (Kotov and Boikova, 2001; Naraki et al., 2013); however, they are based on developmental time rather than morphological landmarks. Such staging systems are not suitable for functional studies since experimental manipulations can cause developmental delays and thus distort the analysis of comparative data sets. We have therefore developed a staging system based on morphological landmarks, which includes 12 embryonic stages (unpublished results). The stages relevant to the segmentation process are briefly described here. Morphological segmentation occurs during stages 6 (naupliar segments) and 7 (postnaupliar segments), which we have further divided into sub-stages depending on the number of segments formed (Fig. 1). These stages correspond to 12–14 h and 15–19 h of development, in the staging scheme established by Kotov and Boikova (2001). However, the first molecular process related to segmentation occured already shortly after gastrulation in stage 4 and we have therefore included the description of stages 4 and 5 here. These early stages have not been included in previous *Daphnia* staging systems (Kotov and Boikova, 2001; Naraki et al., 2013). At stage 4, bilateral half-moon shaped structures called 'Scheitelplatten' can be distinguished anterior to the gastrulation zone, which have been associated with the formation of the eye in other Cladocera (Kühnemund, 1929) (Fig. 1A). At stage 5, a V-shaped area appears that encloses the gastrulation zone and demarcates the area where the naupliar neuroectoderm forms (Fig. 1B). The area is characterised by fewer and scattered nuclei compared to the adjacent medial and lateral areas. At stage 6.1, the second antennal segment (a2) is formed, followed by the simultaneous appearance of antennal segment 1 (a1) and the mandibular segment (md) at stage 6.2 (Fig. 1C and D). The maxillary zone and the first thoracic segment are established at the same time at stage 7.1 (Fig. 1E); however, the separation of the maxillary zone into the first and second maxillary segments (mx1 and 2) occurs in the subsequent stage (7.2), in parallel to the appearance of the second thoracic segment (t2) (Fig. 1F). The remaining thoracic segments (t3 to t5) form sequentially during stages 7.3–7.5 (Fig. 1G–I). The abdominal segments do not form during embryogenesis. Thus the embryonic segmentation processes described here refer to the

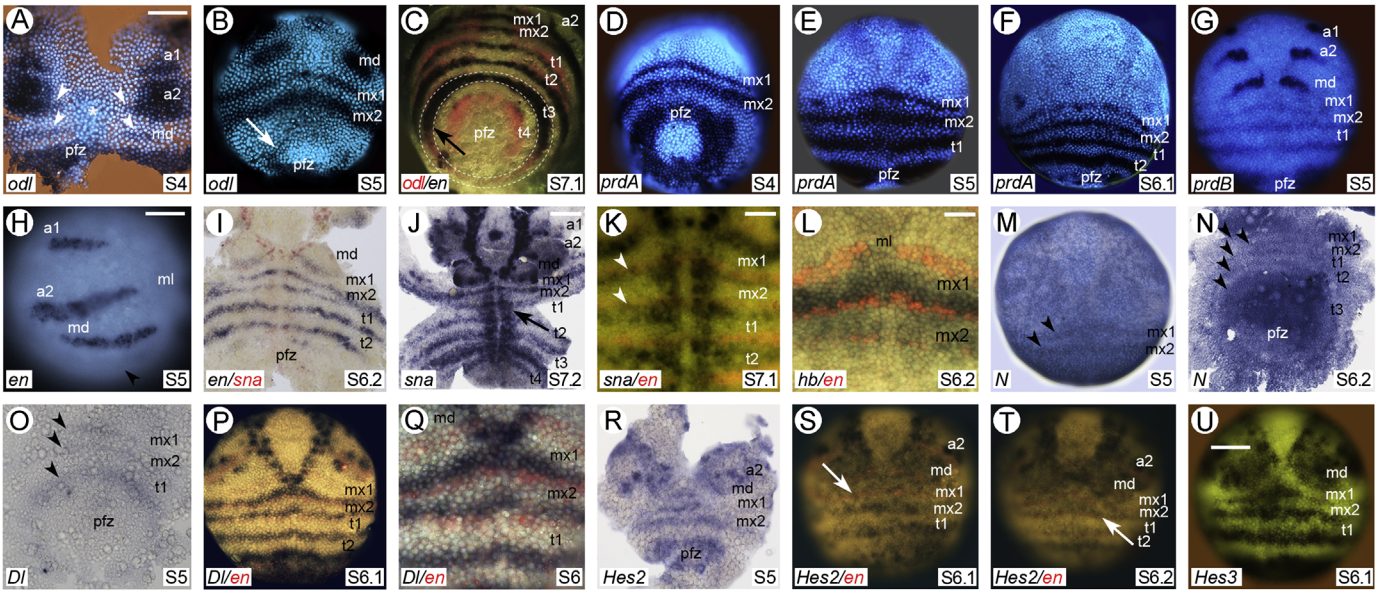

Fig. 2. (A–U) Expression patterns of homologues of the *Drosophila* segmentation cascade and the Notch signalling pathway. Whole mounts (B–H, M, P, and S–U) and flat preparations (A, I–L, N, O, and R) of embryos stained with DIG labelled RNA probes (A, B, D–H, J, M–O, R, T, and U) or double-stained with DIG and FITC labelled RNA probes (C, K, L, P, Q, S, and T). (A) At stage 4, *odl* is expressed in bilateral areas in the head that develops into the first and second antennae and in the postnaupliar formation zone. Faint stripes (arrowheads), which are interrupted by the gastrulation zone (asterisk) are visible. (B) At stage 5, the mx1 and 2 stripes appear and a faint circular *odl* expression domain is visible in the postnaupliar formation zone. (C) The arrow points to the overlapping expression of *odl*/*en*. *En* expression starts later than *odl* in emerging segments and appears first in the ventral and lateral areas before expanding dorsally. Please note that the embryo is slightly tilted to show the posterior formation zone and the circular expression of *odl* in t3 (dashed circles). The posterior part of the circular *odl* expression that corresponds to t4 is not in focus; the expression domain appears therefore horse-shoe-shaped. (D–F) *prdA* stripes are sequentially added in the postnaupliar region. *prdA* is not expressed in stripes in the naupliar area.(G) *prdB* expression in head and trunk. (H–I) *en* expression in a1-md (H: lateral view) and trunk. (J–K) *Sna* expression in transverse stripes and neuroblasts (arrow). *En* and *sna* expressions partially overlap (arrowheads). (L) *hb* expression in mx1/2. (M–U) Striped expression of members of the Notch signalling pathway (arrowheads). *Dl* is expressed anterior to *en*. *Hes* expression partially overlaps with *en* (arrows). *pfz*, postnaupliar formation zone; *ml*, midline. The scale bar in A equates to 100 μm in A–G, I, M, N, P, R, S, and U; the scale bar in H equates to 75 μm in H; the scale bar in J equates to 100 μm in J and the scale bar in K equates to 50 μm in K, O, and T; the scale bar in L equates to 30 μm in L.

formation of the naupliar segments (a1, a2, md) and the embryonic postnaupliar segments (mx1, mx2, t1 to t5). We have named the area, which generates the embryonic postnaupliar segments 'postnaupliar formation zone' (pfz).

We identified homologues of representatives of the different classes of the *Drosophila* segmentation genes (i.e. gap, pair-rule and segment polarity genes) and analysed their expression patterns both in relation to morphological segment formation and the expression of the *D. magna* members of the Notch signalling pathway (see below). The sequence of expression of the *D. magna* pair-rule and segment polarity homologues follows the hierarchical order of the *Drosophila* segmentation cascade, while the gap gene *hunchback* (*hb*) is expressed later than in *Drosophila*. The three *D. magna* pair-rule homologues *odd-skipped-like* (*odl*) and *pairedA/B* (*prdA/B*) are consistently expressed hours before segments become morphologically visible (Fig. 2A–G; Suppl. Fig. 1C–L). At the earliest stage at which metameric stripes could be observed (stage 4), *odl* is expressed in two faint stripes corresponding to a2 and md and in bilateral areas that develop into the 1st and 2nd antennal and mandibular appendages (Fig. 2A). At stage 5, broad stripes of *odl* expression are visible in mx1 and mx2 (Fig. 2B). During stages 6.1–7.2 *odl* stripes corresponding to the 1st to 5th thoracic segments form sequentially. The maxillary as well as the thoracic stripes extend around the embryo so that they form closed circles (Fig. 2C; Suppl. Fig. 1K). *PrdA* and *prdB* show a similar expression pattern to *odl* except that *prdB* is expressed in a1 to md, while *prdA* cannot be detected in these segments (Fig. 2D–G; Fig. 3A; Suppl. Fig. 1C–H). In addition, transcripts of all three pair-rule genes periodically accumulate in the postnaupliar formation zone in concentric rings around the emerging proctodeum (Fig. 4A–H). The circular domain seems to generate the posterior most segmental stripe at a given stage. The stripes do not split and thus do not show double-segment periodicity as in *Drosophila* (Schroeder et al., 2011). *Dam engrailed* (*en*) is expressed at the same time as *prdB* and *odl* in a1 and 2 but follows the pair-rule gene expression in all remaining segments (Fig. 2H and I; Suppl. Fig. 1M and N). *En* expression also extends into the dorsal ectoderm in mx1 to t5 so that the expression domains form closed circles (Fig. 2I; Suppl. Fig. 1A).

We have recently analysed the expression pattern of *Dam snail* (*Dam sna*) in neurogenesis and found that the gene is additionally expressed in transverse stripes (Ungerer et al., 2011). Furthermore, *snail* plays a role in vertebrate mesoderm segmentation and we therefore analysed *Dam sna* expression in relation to the segmentation genes. *Dam sna* is expressed at a short time after *en* in transverse stripes and the genes remain co-expressed throughout (Fig. 2J and K; Fig. 3B; Suppl. Fig. 1O–T). The *sna* stripes abut *en* posteriorly and cover the area where the intersegmental furrows form (Fig. 2K). Neither *en* nor *sna* is expressed in the postnaupliar formation zone. *D. magna hb* is expressed later than the pair-rule

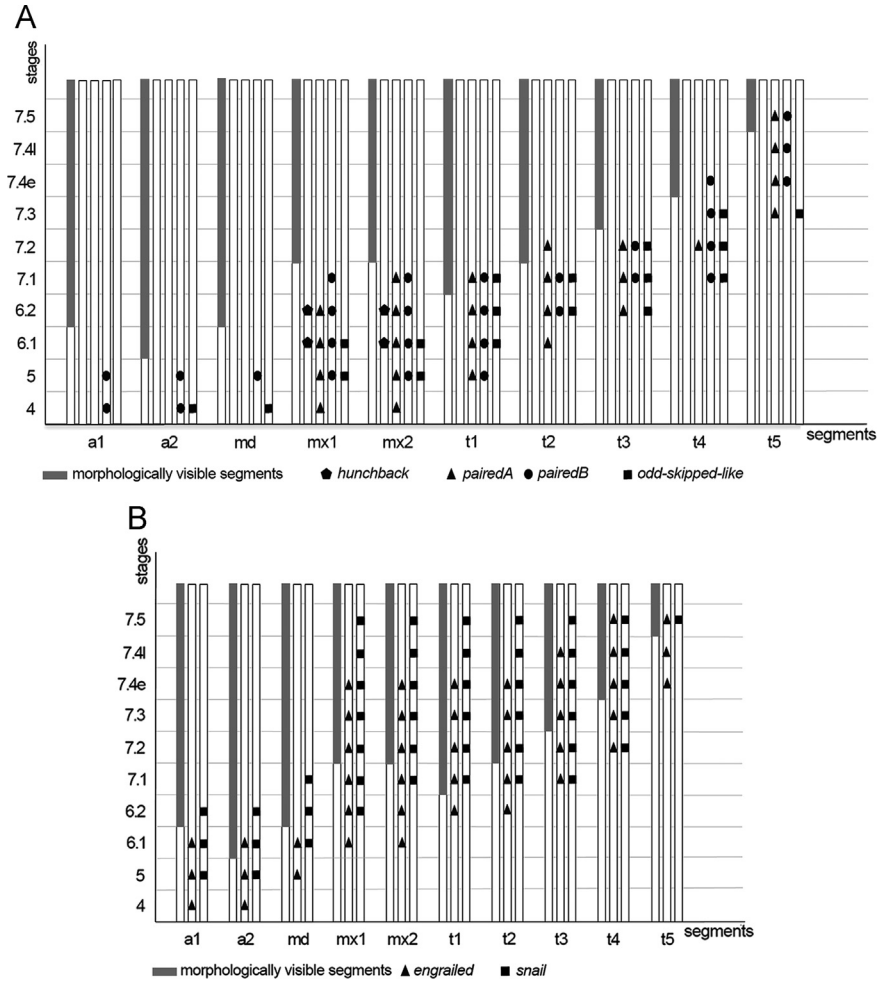

Fig. 3. (A–B) Comparison of the spatial and temporal expression patterns of *D. magna* homologues of the *Drosophila* segmentation cascade. Each block of three and five bars, shows the temporal expression of the respective genes in relation to the appearance of the morphologically visible segments. *Dam hb* shows a restricted transient expression in mx1 and 2. The *D. magna* pair-rule genes are expressed several hours before segments become morphologically visible. For example, in the emerging maxillary segments, *prdA*, *prdB* and *odl* are expressed in stage 5. Intersegmental furrows separate these segments in stage 7.2, i.e. about 9 h after the onset of pair-rule gene expression. (B) *Dam en* is expressed one stage before *sna* in most segments and both genes are expressed before the formation of intersegmental furrows.

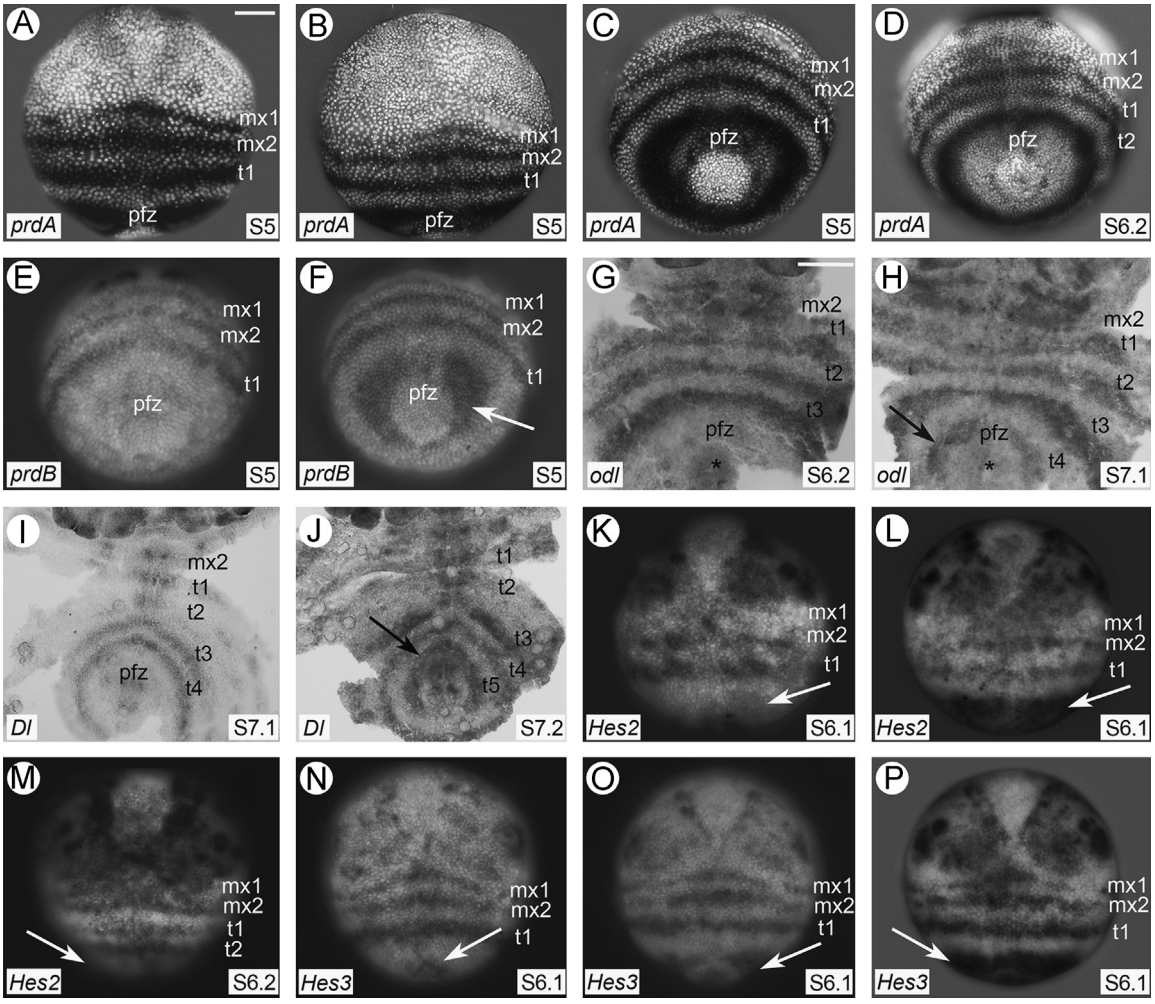

Fig. 4. (A–P) Periodic expression of the pair-rule gene homologues and members of the Notch signalling pathway in the postnaupliar formation zone. Light micrographs of whole mounts and flat preparations of embryos stained with DIG labelled RNA probes of the respective genes. Anterior is towards the top. (A–D) The cyclic expression of prdA in the posterior formation zone correlates with the generation of stripes. Each stripe corresponds to an emerging segment. The stripes narrow as the expression appears more anteriorly. (E and F) PrdB shows the same cyclic expression in the posterior formation zone as *prdA;* however, the overall expression levels of *prdB* in the maxillary and thoracic segments seem to be lower than those of *prdA.* (G and H) *Odl* expression clears away from the posterior formation zone after generation of each stripe. The arrow points to a newly formed stripe. The asterisks indicate the proctodeum. (I, J) Cyclic formation of Dl stripes. The arrow points to the newly formed t5 stripe. (K–P) The Notch effector genes Hes2 and 3 show the same periodic expression in the posterior formation zone as the pair-rule genes. The arrows point to the posterior formation zone, indicating the appearance of periodic gene expression. The scale bar in A equates to 100 μm in A–F and I–P; the scale bar in G equates to 100 μm in G, H.

genes and covers exclusively the maxillary zone. Expression starts at the same time as *Dam en* and before the maxillary zone becomes separated into mx1 and 2 (Fig. 2L; Fig. 3A; Suppl. Fig. 1A and B). The late regional expression excludes the gap gene *hb* from being involved in early steps of segment formation; rather, it might confer regional segment identity.

Notch pathway members are temporally co-expressed with the pair-rule genes

The *D. magna* core members of the Notch signalling pathway—*Dam Delta* (*Dl*), *Notch* (*N*), *Hairy-Enhancer of Split 2* and *3* (*Hes2* and *3*)—are expressed in stripes that encircle the embryo in all nascent segments except for a1 to md. Expression can be detected at the same time as the pair-rule gene transcripts (Fig. 2M–U; Fig. 5; Suppl. Fig. 2). Both the expressions of the pair-rule genes and the members of the Notch signalling pathway are down-regulated shortly before or at the time when intersegmental furrows form. Therefore, there is a brief temporal overlap with *en* expression (Fig. 5A and B). Double-staining shows that the *odl* domain completely overlaps with the *en* stripe and that *Hes2/3* expression slightly overlaps with that of *en* but extends further anteriorly (Fig. 2S and T; Suppl. Fig. 2G, K and O). The *Dl* stripe abuts the *en* stripe anteriorly and therefore overlaps with the *Hes2/3* domain (Fig. 2P and Q). Similar to the pair-rule genes, we observe a periodic appearance of rings of expression in the post-naupliar formation zone for the members of the Notch signalling pathway, which seems to correspond to the consecutive formation of segments (Fig. 4I–P). The expression clears away in the postnaupliar formation zone and the newly formed ring gradually becomes smaller along its anterior–posterior extension as it appears more anteriorly.

Notch function is required at the level of the pair-rule genes

We inhibited Notch signalling by incubating embryos at the beginning of germ band formation in the γ-secretase inhibitor DAPT (Delaune et al., 2007; Pueyo et al., 2008; Ungerer et al., 2012). The embryos (in the following called DAPT embryos) were kept in the solution for 4 h, recovered and allowed to further develop in *Daphnia* medium. Only embryos that showed a partial recovery of segmentation and/or posterior structures were included in the functional analysis thus excluding embryos that

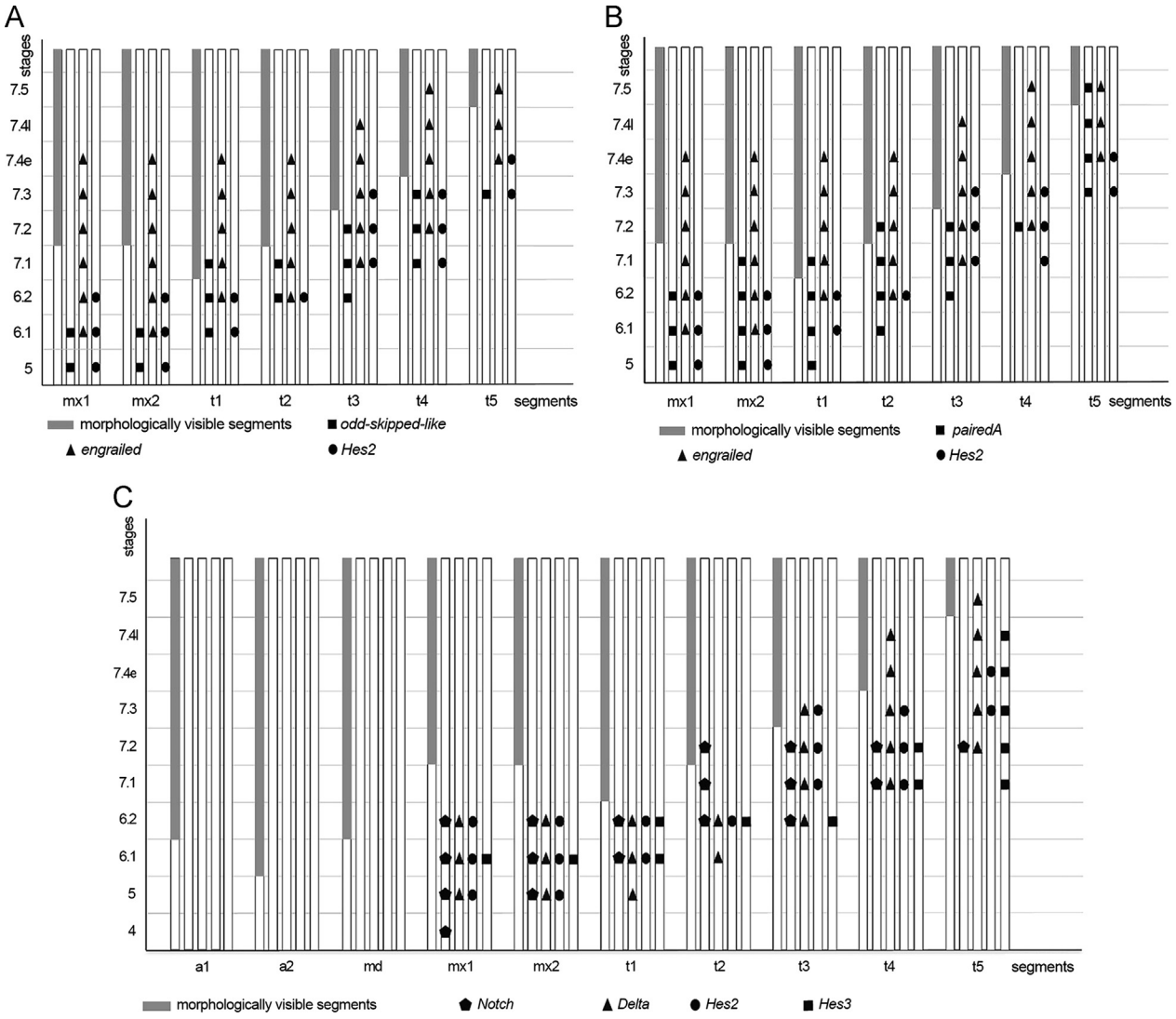

Fig. 5. (A–C) Comparison of temporal and spatial expression of pair-rule, segment polarity and Notch effector genes. Each block of four and five bars, respectively, shows the temporal expression of the respective genes in relation to the appearance of the morphologically visible segments. The members of the Notch signalling pathway are expressed at the same time as the pair-rule genes in the postnaupliar segments (A and B); however, the genes are not expressed in transverse stripes in the naupliar segments (C).

stopped developing. *Dam Hes2/3* expression is severely reduced or absent in DAPT embryos indicating that these genes are targets of the Notch signalling pathway (Fig. 6A and B; Suppl. Fig. 3A–H). While the posterior elongation appears overall normal in DAPT embryos, the anterior–posterior subdivision into segments is affected. In severe phenotypes, affected areas appear completely unsegmented (Fig. 6B, D and D′; Suppl. Fig. 3C and D). This phenotype coincides with the loss of the striped *en* and *sna* expression domains, which normally flank the areas where the intersegmental furrows form (Fig. 6C–I; Suppl. Fig. 3I–P). However, *sna* is strongly expressed in the neuroectoderm of DAPT embryos due to premature differentiation of neural tissue (Ungerer et al., 2012), and *en* expression is either absent or visible in small clusters of neuroectodermal cells (Fig. 6D–I; Suppl. Fig. 3I–P). The phenotype becomes most obvious in partially affected *D. magna* embryos where limb anlagen form but is also reflected in the variable metameric position of residual *en* expression in younger embryos (Fig. 6E–I; Suppl. Fig. 3F, G, K and N–P).

The disruption of Notch function in parcelling the growing germ band into segments can already be seen at the level of the pair-rule genes. Two distinct changes in the *odl* expression pattern can be observed in early DAPT embryos (stage 6.1): the *odl* expressing cells are not tightly packed into stripes and the stripes are not clearly separated and show uneven borders (Fig. 6J–Q). In stage 6.2 additional patterning defects become obvious that affect the dorso-ventral subdivision of the germband into lateral ectoderm (limb anlagen), ventral neuroectoderm and midline in DAPT embryos. In control embryos, the *odl* stripes widen along the anterior–posterior axis in the lateral ectoderm so that they cover the areas where the limb anlagen form. In the medial neuroectodermal area the stripes narrow along the anterior–posterior axis to two to three cell rows. In contrast, in DAPT embryos *odl* expression in the lateral ectoderm is severely reduced or absent at the same stage (Fig. 6N–Q). This altered expression is in line with the absence or malformation of limbs observed in later stages (Suppl. Fig. 3F, G, K and L). *odl* expression is visible in the neuroectoderm of DAPT embryos, although it is irregular which correlates with the size variations of the affected segments. In later stages, the neuroectodermal expression seems to be extended in affected segments compared to control embryos and most likely reflects the neurogenic phenotype described previously (Ungerer et al., 2012). Furthermore the differentiation of the ventral midline is affected. In most cases the midline is expanded along the

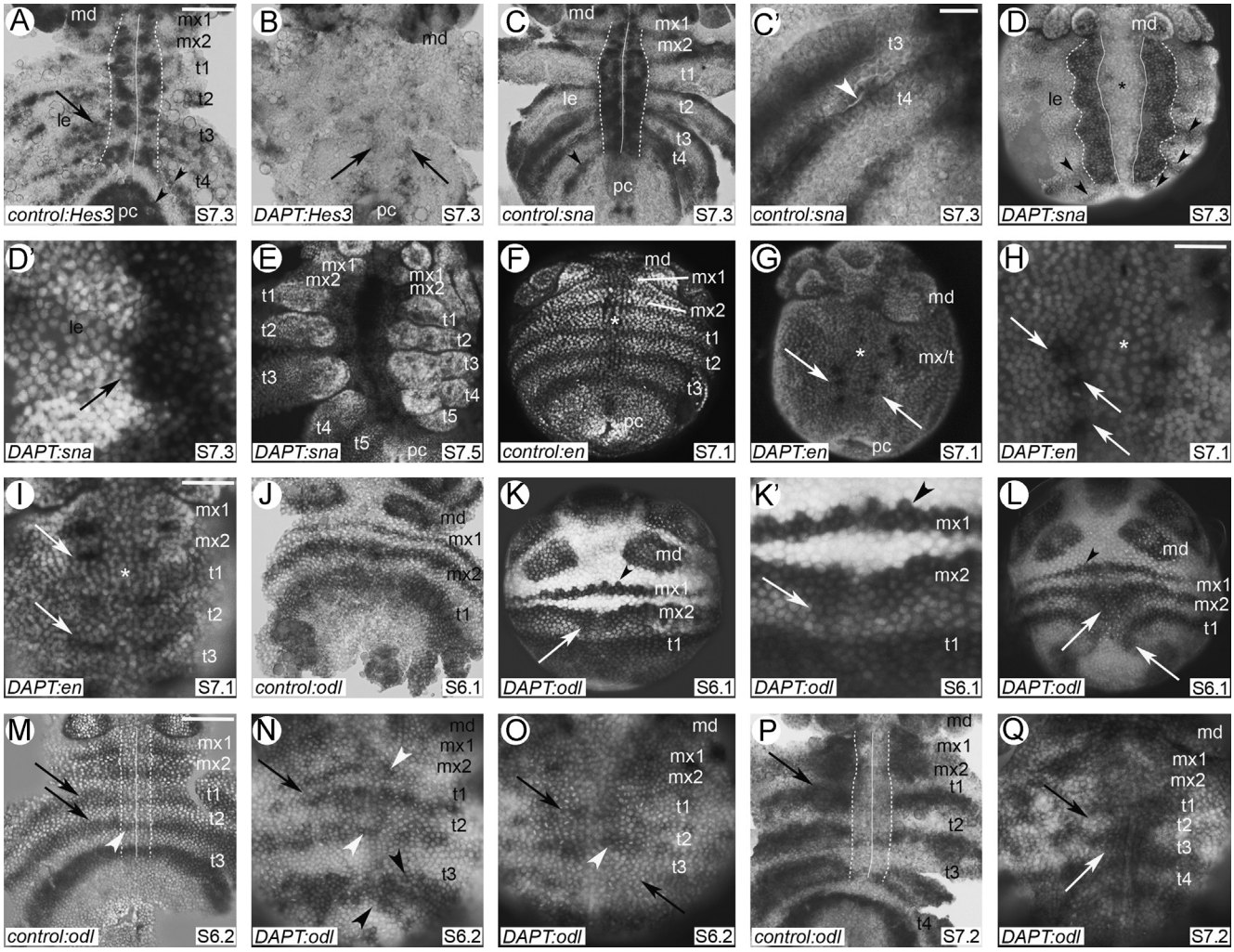

Fig. 6. (A–R) Inactivation of Notch signalling affects segmentation and patterning. We treated 501 embryos with DAPT. 42% of the embryos were not analysable because they were either too young or too old or did not show partial recovery of segmentation and/or posterior structures. 63% of the remaining DAPT embryos showed a specific phenotype as revealed by morphology and changes in the expression patterns of *Dam Hes2* (see Suppl. Fig. 3), *Dam Hes3*, *Dam en*, *Dam odl* and *Dam sna*. (A and C) Control embryos: *Hes3* and *sna* are expressed in stripes (arrowheads) and in the neuroectoderm (dashed lines); solid line: ventral midline. *Hes3* is also expressed in the lateral ectoderm (le) where the limb anlagen forms (arrow in A). (B) Unsegmented DAPT embryo showing low *Hes3* expression posteriorly (arrows). (C′) Same embryos as in C. High magnification of *sna* expression surrounding the intersegmental furrow (arrowhead). (D) Strong neuroectodermal *sna* expression (dashed line) in unsegmented DATP embryo; stripes appears posteriorly (arrowheads). (D′) Same embryos as in D. High magnification of *sna* expression in the neuroectoderm (arrow). Intersegmental furrows are absent in the lateral ectoderm. (E) The DAPT embryo shows an irregular segment size on the left side, which is reflected in the irregular size of the limb anlagen. (F) Control embryo showing regular transverse *en* stripes in mx1 to t3. The asterisk indicates the ventral midline. (G–I) *en* is expressed in irregular clusters in DAPT embryos (arrows). The asterisks indicate the expanded ventral midline. The midline cells lack the typical morphology and arrangement (compare to F and Suppl. Fig. 3J). (J) In the control embryo, the *odl* stripes are clearly separated and show smooth borders. (K, K′–L) In DAPT embryos the clear separation of the *odl* stripes is affected (white arrows) and the borders of the stripes are uneven (black arrowheads). K′ is a high magnification of K. In the embryo in L the separation of the stripes is only affected in the ventral-medial area. (M) At stage 6.2, the shape of the *odl* stripes changes in control embryos. In the lateral ectoderm the *odl* stripes broaden along the anterior–posterior axis so that they cover the area where the limb anlagen appear (black arrows). In the medial neuroectodermal area enclosed by the dashed lines, the *odl* stripes narrow along the anterior–posterior axis and cover about two to three cell rows (white arrowhead). (N and O) In the DAPT embryos patterning is affected along both axes. *Odl* expression is severely reduced in the lateral ectoderm where the limb anlagen normally form (black arrows) and the expression is irregular in the ventral neuroectoderm (white arrowheads). The black arrowheads point to the uneven borders of the t3 *odl* stripe. (P) At stage 7.2 the neuroectodermal *odl* expression is down-regulated in the maxillary and first thoracic segments in the control embryo, while the gene is strongly expressed in the limb anlagen. (Q) *Odl* expression is extended in the medial area of the DAPT embryo and the expression domains of t2 and t3 are not separated (white arrow). This altered expression might reflect the neurogenic phenotype described previously (Ungerer et al., 2012). *Odl* expression in the lateral ectoderm is severely reduced (black arrow). Asterisks: midline area; le, lateral ectoderm; pc: proctodeum. The scale bar in A equates to 100 μm in A–C, D, E–G, J, K, and L; the scale bar C′ equates to 30 μm in C′; the scale bar in H equates to 50 μm in H, K′; the scale bar in I equates to 40 μm in I; the scale bar in M equates to 100 μm in M–Q.

dorso-ventral axis and the ventral midline cells lose their typical cuboid shape, indicating a differentiation defect (Fig. 6D; Suppl. Fig. 3C, J and K).

Discussion

Here we analyse for the first time the embryonic patterning mechanisms underlying segmentation in the crustacean *D. magna* using molecular markers. We show that the *D. magna* pair-rule and segment polarity genes follow the same hierarchical pattern of expression as in *Drosophila* in relation to segment formation; however, the pair-rule genes are expressed in every segment rather than showing double-segment periodicity as in *Drosophila* (Schroeder et al., 2011). In addition, the anterior border of the *D. magna* pair-rule genes is shifted from md to a1 compared to *Drosophila,* if we assume homology of the individual head segments in insects and crustaceans (Schroeder et al., 2011). The late

regional expression of the gap gene *Dam hb* suggests a role in segment identity in mx1 and 2 in *D. magna*, rather than an involvement in early segmentation. A similar function in regional identity has been described in *Drosophila* and *Tribolium* and might therefore represent an ancestral feature of pancrustacean segmentation (Lehmann and Nüsslein-Volhard, 1987; Marques-Souza et al., 2008).

The role of Notch signalling in D. magna segmentation—two possible scenarios

Functional data on Notch signalling in insects and spiders (chelicerates) as well as expression studies in a centipede hint at similar mechanisms of segmentation in vertebrates and arthropods (Chipman and Akam, 2008; Pueyo et al., 2008; Stollewerk et al., 2003). It is generally assumed that the involvement of Notch in arthropod segmentation is linked to the presence of a segmentation clock, which drives the cyclic expression of segmentation genes (Chipman and Akam, 2008; Pueyo et al., 2008). However, until today there is no direct evidence for Notch regulating gene oscillation in nascent segments in arthropods, although the presence of a segmentation clock involving *Tc-odd-skipped* has recently been demonstrated in the posterior growth zone of an euarthropod, the insect *Tribolium castaneum* (Sarrazin et al., 2012). Furthermore, the pleiotropic functions of Notch in segmentation, patterning and organ formation complicate the interpretation of functional data in relation to segment formation. For example, early functions of Notch in establishing the posterior growth zone might obscure later functions in segmentation as has been shown in the spider *Parasteatoda tepidariorum* (Oda et al., 2007). On the other hand, inhibition of later functions of Notch might result in loss of segments and/or termination of development although the function is not linked to segment formation per se (Kainz et al., 2011).

To overcome these difficulties we transiently inhibited Notch signalling at the start of segmentation and only included embryos in the functional analysis that showed partial recovery of metameric patterns in the posterior germband. This enabled us to unambiguously show that the loss of Notch function correlates with a loss of segmentation and is not due to a developmental arrest. The formation of the naupliar segments (a1, a2, md) was not affected although defects could be observed in the antennal and mandibular limbs in later stages. Our data show that Notch signalling influences segmentation at the level of the pair-rule genes in the postnaupliar segments of *D. magna*. Furthermore, the changes in the expression pattern of the pair-rule homologues *odd-skipped-like* suggest a potential link between the Notch effector genes *Hes2/3* and the *Drosophila* segmentation cascade.

Two scenarios are conceivable for explaining the role of Notch signalling in segment formation in *D. magna* (Fig. 7). (1) If we apply the vertebrate model (Richmond and Oates, 2012) and assume the presence of a segmentation clock involving Notch signalling as has been suggested for other arthropods (Chipman and Akam, 2008; Pueyo et al., 2008), the Notch effectors Hes2/3 might synchronise the spatio-temporal expression of segmentation genes including *odl* (or genes that directly regulate *odl*) (Fig. 7A). This assumption is supported by the periodic expression of the pair-rule genes and the members of the Notch signalling pathway in concentric rings in the posterior growth zone. Furthermore, similar aspects of segmentation seem to be affected in *D. magna* and vertebrates, including fuzzy segmental borders and variations in segment size, as these phenotypes have also been reported in somites of *Hes* mutant mouse embryos (Del Barco Barrantes et al., 1999). Interestingly, the possible involvement of Snail in segment border formation in *D. magna* represents an additional similarity in crustacean and vertebrate segmentation. In vertebrates *snail* expression oscillates mainly in phase with Notch signalling members in the presomitic mesoderm, although its oscillation is independent of Notch (Dale et al., 2006). Down-regulation of *snail* by FGF and Wnt signalling is required for transforming the anterior presomitc mesoderm into epithelial cells

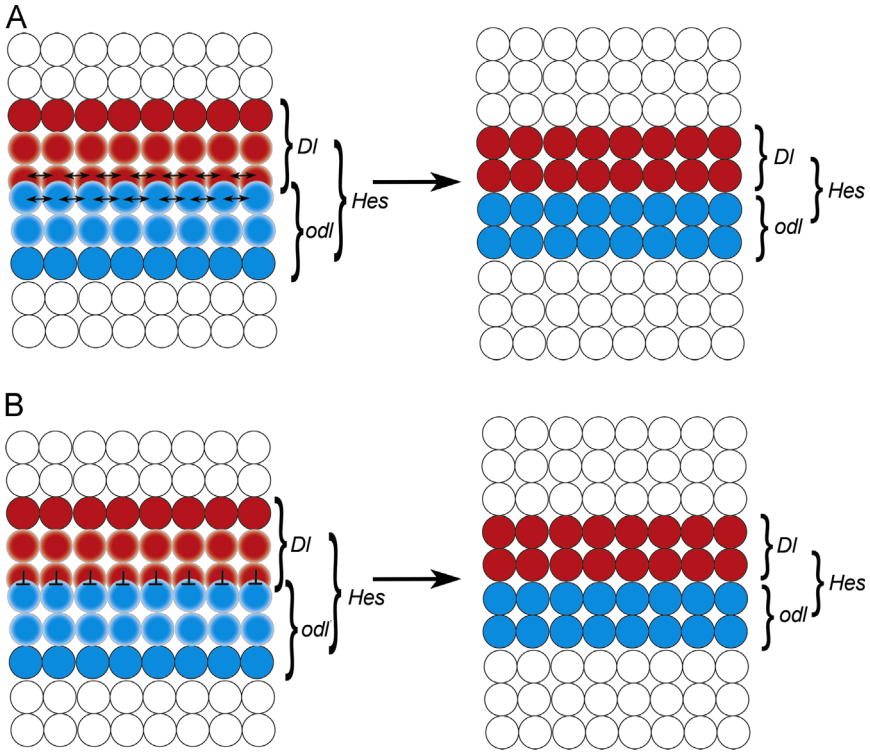

Fig. 7. (A–B) Two possible scenarios of Notch function in the cellular process of segmentation in *D. magna*. (A) In the first scenario, there is initially an overlap of *Dam Dl*, *Dam odl* and *Dam Hes* expression in the postnaupliar formation zone. Notch signalling synchronises gene oscillation in emerging segments and generates distinct smaller stripes of gene expression. (B) In the second scenario, there is also an overlap of *Dam Dl*, *Dam odl* and *Dam Hes* expression but Notch activity is strongest at the border of *Dam Dl* expressing and non-expressing cells. *Dam Hes2* and *3* repress *odl* expression so that a distinct stripe of *odl* expression is formed.

eventually resulting in the formation of somites. The co-expression of *Dam sna*, *Dam Hes2/3* and *Dam en* around the area where the intersegmental furrows form as well as the collective absence of expression in areas devoid of segmental borders in *D. magna* DAPT embryos strongly suggest a role of *Dam sna* in segmentation. However, despite the involvement of *snail* in both species, neither the underlying cellular processes nor the molecular interactions seem to be comparable in *D. magna* and vertebrates.

(2) The second scenario is based on the known function of Delta and Notch in generating sharp boundaries and is also in line with the DAPT phenotype (Rauskolb et al., 1999). In this scenario, *Dam Dl* would activate Notch signalling most strongly at the border of *Dam Dl* positive and *Dam Dl* negative cells (Fig. 7B). This area corresponds to the anterior border of *odl* expression. The Notch effectors *Dam Hes2/3* would directly or indirectly repress *odl* expression and thus generate the sharp anterior boundary of the metameric *odl* domains. Interestingly, interactions of Notch signalling and members of the Odd-skipped family in boundary formation have been described in *Drosophila* limb segmentation (Hao et al., 2003). Furthermore, in this scenario the Notch effector genes would be classified with pair-rule genes and contribute to restricting the initially broad pair-rule domains to small segmental stripes. Thus the cause for the DAPT phenotype would be the misexpression (deregulation) of pair-rule genes. This conclusion is supported by the irregular expression of *Dam odl* in DAPT embryos but also by data from *Tribolium* showing that loss of function of the primary pair-rule genes *odd-skipped*, *even-skipped* and *runt* leads to either loss or ectopic expression of other pair-rule genes and results in unsegmented embryos (Choe et al., 2006).

Within insects a requirement for Notch signalling in segment formation has been demonstrated in the basal insects *Periplaneta americana* and *Gryllus bimaculus* (Mito et al., 2011; Pueyo et al., 2008). However, an involvement of Notch signalling in segmentation in *G. bimaculus* has been challenged by the Extavour group (Kainz et al., 2011), who demonstrated that the segmentation phenotype results from developmental delays and cell specification errors, rather than from a direct function of Notch signalling in segmentation. The identical spatio-temporal expression patterns of *Dl*, *hairy* and *en* and the down-regulation of *hairy* following Notch inactivation in *P. americana* (Pueyo et al., 2008) and *D. magna* point towards similar functions of Notch in insect and crustacean segmentation. Interestingly, in *Drosophila* the pair-rule gene *hairy* has as a function similar to the one suggested in scenario 2 in repressing *odd-skipped* expression during embryonic segmentation, although independently of Notch (Jiménez et al., 1996). It is tempting to speculate that *hairy* regulation in the segmentation process has been uncoupled from Notch signalling in insect lineages that do not require the signalling pathway in segmentation.

Evolutionary variations in the requirement of Notch along the anterior–posterior axis

Depending on the mode of development (short, intermediate or long germ) a variable number of anterior segments is prepatterned in the blastoderm of arthropods and it seems that Notch signalling is only required for the formation of those segments that are generated by the posterior growth zone (Oda et al., 2007; Pueyo et al., 2008). Since the number of segments generated simultaneously in the blastoderm varies, the anterior border of Notch requirement in segmentation is also variable but usually involves trunk segmentation and in particular abdominal segmentation. It has been suggested that *Daphnia* and other water fleas show long germ development, i.e. that segments are formed simultaneously (Schwartz, 1973). However, our data do not support this assumption. While the naupliar segments are formed almost simultaneously, there is clearly a delay between the formation of the naupliar and postnaupliar segments. Furthermore, the postnaupliar segments are generated in an anterior to posterior sequence except for the late subdivision of the maxillary region. The difference between naupliar and postnaupliar segments is also reflected in the requirement of Notch signalling since Notch inactivation does not affect naupliar segmentation. Interestingly, in *D. magna* the Notch segmentation area includes the two posterior gnathal head segments—mx1 and mx2—while in the insect *P. americana* the anterior-most segment affected by Notch inactivation corresponds to the first thoracic segment (Pueyo et al., 2008). Given the correlation between the different modes of segment formation (simultaneously in the blastoderm versus sequentially from a posterior growth zone) and Notch requirement in segmentation, we suggest that the maxillary segments as well as all trunk segments are generated by the posterior growth zone in *D. magna*. Although Notch signalling seems to be required for segments that are generated by the posterior growth zone, the pathway is not involved in elongation of the germ band. DAPT treatment does not result in shorter embryos; rather, the cellular material is present but not subdivided into segments. This is again in contrast to Notch inactivation in *P. americana* which results in truncated embryos (Pueyo et al., 2008).

In contrast to *D. magna* embryos, Notch inactivation in larvae of the branchiopods *Thamnocephalus platyurus* and *Artemia franciscana* does not affect segmentation up to the second or third thoracic segment (Williams et al., 2012). However, in these branchiopods all trunk segments form during larval stages and it is possible that different mechanisms operate in larval and embryonic segmentations. This is supported by the fact that the authors did not detect any additional phenotypes in DAPT larvae. Given the pleiotropic functions of Notch in arthropod embryos, the molecular mechanisms of larval and embryonic segmentation might therefore not be directly comparable.

Notch signalling is also involved in patterning the dorso-ventral axis

In addition, we have shown here that Notch signalling is not only involved in patterning the anterior–posterior axis but also the dorso-ventral axis. In the lateral ectoderm the limb anlagen are partially missing and both the neuroectoderm and the midline are expanded. Midline cells lose their characteristic morphology indicating that their differentiation is affected. Similar midline phenotypes have been reported in functional studies on Notch signalling in the *Drosophila* midline (De Renzis et al., 2006; Menne and Klambt, 1994). Notch requirement in the development of the ventral midline might therefore be an ancestral feature of pancrustaceans. An involvement of Notch signalling in limb and nervous system development has also been demonstrated in insects and chelicerates and therefore seems to be an ancestral function of Notch in euarthropods (Hao et al., 2003; Kunisch et al., 1994; Prpic and Damen, 2009; Stollewerk, 2002).

Conclusions

Notch signalling is required for the formation of postnaupliar segments in *D. magna*. The irregular expression of *odd-skipped-like* in DAPT embryos suggests that Notch is involved in early steps of segmentation at the level of the pair-rule genes. The functional data invite two alternative interpretations regarding the cellular processes of Notch involvement. Notch signalling might synchronise gene oscillation in nascent segments and thus might function in a mechanism comparable to vertebrate mesoderm segmentation. Alternatively, Notch signalling might not be a part of segmentation clock in *D. magna* (and possibly in arthropods in

general) but the Notch effector genes might classify with the pair-rule genes and function in restricting the expression domains of other segmentation genes.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.09.021.

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